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The major objective of this project is to establish a new modality for the treatment of breast cancer that employs the combination of chemokine gene-modified fibroblasts with breast tumor-pulsed dendritic cells (DC) to both recruit and/or concentrate from the periphery low frequency immune reactive T cells as well as to potently stimulate these effector cells once localized at the vaccination site. During the first year of this four-year project, studies focused on two major areas specified in the Statement of Work: 1) to optimize human DC generation and function; and 2) to construct expression vectors containing chemokine cDNAs for subsequent gene transfer into fibroblasts. Marked enhancement of human peripheral blood-derived DC number and function (i.e. presentation of tetanus and candida antigens as well as stimulation of primary allogeneic mixed leukocyte response) could be achieved by the addition of TNF-α to cultures containing the combination of GM-CSF and IL-4. DC obtained from advanced breast cancer patients could potently stimulate the generation of autologous, KLH-specific $CD4^+$ T cells in vitro. Construction of retroviral vectors based on the MFG backbone and production of retroviral supernatants at high titer by a transient packaging cell system have been accomplished, which have resulted in successful chemokine (i.e. RANTES, Lymphotactin, MIP-1β) gene transfer into fibroblasts. Collectively, the data provided in this annual report demonstrate successful completion of studies according to the proposed year 1 timeline in the grant application.

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James F. Arnold 07/28/97

PI - Signature Date

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Introduction

Immunotherapies that have employed the adoptive transfer of lymphokine-activated killer (LAK) cells, tumor infiltrating lymphocytes (TIL), or tumor draining lymph node (TDLN) cells in combination with the systemic administration of interleukin-2 (IL-2) have resulted in the regression of several types of tumors in both humans and animals (1-4). In certain patients, responses have been observed in both renal cell carcinoma and melanoma. Few attempts, however, have been made to utilize this form of therapy for tumors of other histologic types, including those most prevalent in the overall patient population and often considered to be "poorly immunogenic". Breast cancer is an histology that falls within this category.

It has been shown that T cells from some patients with breast cancer can specifically react to autologous tumor as measured in vitro by either cytotoxic activity or the release of cytokines (5-8). This finding has recently led to the molecular identification of breast cancer-associated antigens or peptides, e.g., MUC-1 (6,7) and HER2/neu (8), that are recognized by autologous T cells from cancer patients. Although this rather new information raises the intriguing possibility for immune interventions in the treatment of breast cancer, the overall level and incidence of detectable T cell responses in these patients are rather low. This limitation could be due, in part, to: a) tumor-induced, active immune suppression (9) and/or defects in T cell signaling (10) in the cancer patient, which may actively down regulate the functional activity of antitumor effector cells. Recent data in rodents suggest that the latter deficit may be overcome by immunization with tumor cells that have been molecularly-engineered to secrete IL-2 (11); b) low incidence of antigen-reactive T cells. Although precursor CTL with activity against whole tumor cell targets or purified tumor peptides have been detected in peripheral blood and lymphoid tissue of some patients, by limiting dilution analyses their frequency is either absent or difficult to detect (12); and c) failure of host-derived T cells to recognize antigens on the tumor cell surface itself. In this regard, we have described significant inherent defects in antigen processing and presentation by certain human solid tumors that could be a crucial mechanism for their inability to stimulate the afferent arm of the immune response (13).

Recent attempts to improve upon immunotherapy for human cancers include the genetic-modification of TIL to express exogenous genes encoding for antitumor cytokines (1-2, 14) or new "chimeric" receptor genes to redirect tumor antigen specificity (15), as well as the utilization of CTL-defined tumor peptides (7,8,16) and gene-modified tumor cells (17-19) as immunogens to generate more potent TIL or TDLN or to impact directly on established metastatic disease by serving as "therapeutic vaccines". We have been investigating novel approaches to enhance the activity of tumor vaccines in order to increase the frequency of tumor-reactive T cells, to overcome tumor-induced immune suppression, and to stimulate potent primary and secondary responses to poorly-immunogenic tumors. Our recent studies have involved the use of either dendritic cells (DC) as potent antigen presenting cells (APC) or certain chemokines as potent immune cell chemoattractants.

Molecularly-engineered tumor cell vaccines have been shown in some published reports to be effective in reducing the size of preestablished tumor masses in rodents, but the issue of potency of tumor vaccination has become an important one. Most of the human clinical trials currently underway in gene therapy for a variety of different cancers involve introducing one of several cytokine genes into either tumor cells or autologous fibroblasts. The rationale for these trials is that, when reintroduced, these genetically modified cells will serve as sites of cytokine production and thus enhance immunity by several different mechanisms (depending on the cytokine of choice). To date, these include IL-2, IL-4, IL-7, IL-12, TNF-α, IFN-γ, and GM-CSF. However, the ability to induce tumor regressions or inhibit metastases by cytokine/tumor vaccines has been shown in a number of preclinical animal studies to be overcome by larger tumor cell inocula or by prolonging the period of time between tumor establishment and subsequent tumor/cytokine vaccination.

DC are highly potent APC of bone marrow origin (20,21), which have been shown to stimulate both primary and secondary T and B cell responses (22,23). Animal studies have indicated that DC are preferentially responsible for sensitization of naive T cells in their first exposure to antigen (24). Antigen distribution in

the host environment often favors uptake and presentation by DC rather than macrophages or B cells (25), and subsequent migration of primed DC to lymphoid organs enhances targeted presentation of antigens to the immune system (26). Antigen-pulsed DC have been used successfully in culture to sensitize naive mouse $CD4^+$ T cells to a variety of antigens (27). When DC are cultured with exogenous proteins in vitro, presentation of antigen in a major histocompatibility (MHC) Class II context is favored (28), but a variety of treatments including peptide pulsing enables cultured DC to present antigen in a MHC Class I context as well (28,29). In addition, tumor-pulsed DC-rich preparations have been used successfully to treat established mouse tumors in vivo (30-32).

That MHC Class II-bearing APC primarily stimulate $CD4^+$ T cells is of particular interest, since in several murine tumor models antitumor $CD4^+$ T cells have proven capable of mediating tumor rejection or conferring protective immunity (33-35) and a human $CD4^+$ T cell defined tumor antigen has been recently defined (36,37). In the animal models, the successful culture of antitumor $CD4^+$ T cells has relied on immunization of rodents against a tumor or purified tumor protein and subsequent in vitro restimulation of sensitized T cells with macrophages or spleen cells pulsed with a purified tumor protein (34-38). Such strategies depend upon the availability of purified proteins; however, specific tumor rejection antigens are not yet known for many human tumors, including those of breast origin. Furthermore, the study of $CD4^+$ T cells has been impeded because of the difficulty in expanding these cells in vitro. In contrast, it has proven far easier to expand antitumor $CD8^+$ T cells, such as in the majority of human and mouse TIL studies. Recently, however, we have successfully developed a culture system to study the properties of antitumor $CD4^+$ T cells that employ DC as APC. Our data have shown that relatively crude membrane preparations of tumor cells will suffice as sources of tumor antigen, avoiding the necessity for molecular identification of the tumor antigen for effective immunization (39-41). We have now identified for the first time specific $CD4^+$ T cell reactivity to tumor cells both in the mouse and human (39-41). Moreover, methods are now available to generate sizable numbers of highly-enriched DC, both in humans and in rodents, by culturing progenitor cells in the presence of GM-CSF, TNF-α, and/or IL-4 (42-48). The establishment of DC cultures from the peripheral blood of adult patients has raised the very important possibility of now using these cells as immunotherapeutic agents for the treatment of breast cancer (30).

With respect to cytokine gene-modified tumor cells, much of the work to date has employed interleukins, interferons, TNF-α, and hematopoietic colony stimulating factors (17-19,49). Another class of cytokines which has more recently received attention, is the chemokines (or chemoattractant cytokines). Chemokines are essential for leukocyte trafficking and inflammatory processes and share structural similarities, including four conserved cysteine residues which form disulfide bonds in the tertiary structures of the proteins (50). Traditionally, the chemokine superfamily has been divided into two subgroups: C-X-C (where X is any amino acid) and C-C, according to whether an intervening residue space the first two cysteines in the motif (50). This structural distinction has been shown to delineate a general distinction in the biological properties of these molecules: most C-X-C chemokines are chemoattractants for neutrophils but not monocytes, whereas C-C chemokines appear to attract monocytes but not neutrophils (50). Of importance, the C-C group has also been shown to be chemoattractant to lymphocytes. For example, the C-C chemokine RANTES is a chemoattractant for memory T cells in vitro (51) and human macrophage inflammatory proteins-1α and -1β (MIP-1α, MIP-1β) have been found to be chemoattractant for distinct subpopulations of lymphocytes including naive T cells and B cells (52). Recent evidence suggests that the C-C chemokine MCP-1 induces T cell migration as well (53). It should also be noted that natural killer (NK) cells migrate vigorously in response to RANTES, MIP-1α, and MCP-1 (50). In addition to chemoattraction, RANTES has been recently shown to activate T cells (54,55), and many of the C-C chemokine members increase the adhesive properties of the cells for which they are chemoattractant (50,56). The discovery of a new protein suggests that the superfamily now has an additional branch, the 'C' branch. Lymphotactin, a molecule isolated from pro-T cells, clearly lacks the first and third cysteines in the four cysteine motif, but shares a great deal of amino acid similarity at its carboxyl terminus with C-C chemokines (57,58). Of importance, lymphotactin is the only superfamily member to date to be selectively chemotactic to lymphocytes only, as it does not attract either monocytes or neutrophils (57,58).

We have examined one of the C-C chemokines RANTES for its properties in vivo using a murine tumor model (59). We have shown that immunogenic murine tumor cells that stably produce human RANTES chemokine after gene-modification lose their ability to form solid tumor masses in vivo. Furthermore, this loss of tumorigenicity correlates with in vitro chemoattraction of tumor-specific T cells and appears to be mediated in vivo by various host-derived immune cells since the tumorigenicity of RANTES-secreting cells is restored when $CD8^+$ and $CD4^+$ T cells are depleted or when murine macrophage migration is inhibited (59). Thus, this study represents the first analysis of the functions of RANTES as produced from an in vivo source, and shows that the chemoattractant properties of this chemokine for monocytes and T cells as predicted from in vitro assays using human cells appear to be broadly relevant in this in vivo murine model. Other C-C chemokines, namely MCP-1 and murine TCA3, have been recently shown to inhibit in vivo tumor growth as well (50, 60).

Given this background, this funded research proposal focuses on a series of studies to determine whether molecules potently and selectively chemotactic for naive and memory T cell subsets can be used in conjunction with tumor-pulsed DC to provide a highly effective means of both detecting and augmenting the immune response to breast cancer.

Technical Objectives and Timelines

The following Technical Objectives and their corresponding timelines were specified in the original funded grant application:

1. To evaluate the capacity of human dendritic cells to detect T cell specific responses to autologous breast tumor in vitro (Months 1-48).

2. To generate high, stable chemokine producer cells by the introduction and expression of the relevant genes in human fibroblast preparations (Months 1-36).

3. To determine the capacity of the combination of chemokine-secreting cells and dendritic cells pulsed with autologous breast tumor to detect, attract, and augment specific, antigen-reactive T cells in vitro (Months 12-48).

Body (Methods, Results, Discussion)

The research conducted during the first year of this four-year award concentrated mainly on experiments proposed in Technical Objectives 1 and 2, with studies proposed in Technical Objective 3 to be initiated in year 2 of the award. Therefore, the laboratory effort has attempted to cohere to the original timetable (i.e. Statement of Work) provided in the grant application. All data figures and a table referred to in the text below are provided in the Appendix section of this annual report.

(a) Technical Objective 1: Much of the proposed studies in this aim was focused on efforts to first optimize the generation/production of human dendritic cells (DC) to serve as potent antigen presenting cells (APC) in order to best detect and enhance low level specific T cell responses in vitro. This issue is a critical one, since breast tumors are considered to be poorly-immunogenic and, therefore, utilizing the most potent DC is paramount to being able to detect the stimulation of autologous, breast cancer-specific T cells. Because it has been shown that the level of "maturity" of peripheral blood DC can have demonstrable effects on the capacity of these cells to effectively present antigen(s) and trigger primary or secondary immune T cell reactivity, we had proposed to compare in our experiments three separate preparations of DC derived in the presence or absence of cytokines. Thus, in Technical Objective 1, we compared "cytokine-driven" vs. "fresh" DC. Standard, well-established procedures were applied for the separation of enriched

DC from human peripheral blood and their subsequent pulsing with defined antigens (41,43,48,61-63). Following initial attempts to obtain sufficient numbers of "fresh" DC for exhaustive phenotypic and functional analyses, it became clear that this population provided an inadequate yield because "fresh" DC comprised only a very small percentage of the whole peripheral blood collections. Therefore, we have abandoned the study of "fresh" DC for practical and logistical reasons. Rather, **we have now completed optimization studies of the generation of "cytokine-driven" (GM-CSF plus IL-4) human DC obtained from peripheral blood for yield, purity, phenotype, and function.** Before optimization studies were begun, we evaluated the ability of peripheral blood-derived T cells from advanced breast cancer patients to respond to defined antigens [tetanus toxoid and Keyhole limpet hemocyanin (KLH)] that were pulsed onto DC generated from 7-day GM-CSF- and IL-4-supplemented cultures. $CD4^+$ T cells or unfractionated T cells from two different advanced breast cancer patients showed potent and specific proliferative responses to autologous DC pulsed with either tetanus, after a single (Figure 1) in vitro stimulation, or to the poorly-immunogenic antigen KLH, after three (Figures 2 and 4) but not one (Figures 1 and 3) in vitro restimulations (Figures 2 and 4). **These results demonstrate that T cells obtained from advanced breast cancer patients can be "educated" in vitro to respond strongly to a poorly-immunogenic, well-defined antigen (i.e. KLH) only when presented by autologous "cytokine-driven" DC. Moreover, the studies show that it is possible to obtain functional DC from the peripheral blood of advanced breast cancer patients that have failed multiple standard therapies.**

We next performed a series of experiments to optimize the generation/production and further enhance the functional activity of DC. We examined the effect(s) of a variety of different recombinant cytokines (and concentrations) added (at different time points) to our standard 100 ng/ml GM-CSF and 50 ng/ml IL-4 "cytokine-driven" DC cultures. Our screening of recombinant cytokines revealed that TNF-α (10 ng/ml) mediated the most potent activity, particularly when added to 7-day GM-CSF/IL-4-containing DC cultures and when allowing these cultures to continue for another 7 days after its inclusion (i.e. 14-day DC cultures). As shown in Table 1, the addition of TNF-α resulted in at least a two-fold increase in the overall number of DC recovered from 14 day cultures. As shown in Figure 5, FACS analyses also revealed enhanced expression of the critical co-stimulatory molecules CD80 and CD86 (black color histograms) in the TNF-α supplemented DC cultures compared to the non-supplemented ones (white color histograms). Most importantly, the addition of TNF-α resulted in substantial augmentation of several distinct functions of human peripheral blood-derived DC. TNF-α-treated DC from three different donors consistently showed a dramatic enhancement in the priming/stimulation of naive T cells in a 5-day primary allogeneic mixed leukocyte reaction (Figures 6 and 7). These former cells were also capable of mediating extremely potent APC activity of tetanus toxoid and candida albicans antigens to autologous T cells in 5-day proliferation assays (Figures 8 and 9). In this regard, the addition of TNF-α allowed for a significantly fewer number of DC to elicit T cell proliferative responses (Figure 9), with a concurrent dramatic reduction in the concentration of antigen(s) required for pulsing of DC to serve as effective APC (Figure 8). Similar attempts to mimic the effects of TNF-α on "cytokine-driven" DC by triggering with monoclonal antibodies to other members of the NGF receptor family, namely anti-CD27, anti-CD30, and the combination of anti-CD27 and anti-CD30, were not effective (not shown). **The importance of these findings centers around the theoretical possibility that antigen(s) expressed by human breast cancer cells at very low amounts on the cell surface may now be effectively presented to autologous T cells when pulsed onto GM-CSF/IL-4 "cytokine-driven" DC that have been additionally treated with TNF-α.** [Similar studies utilizing actual patient-derived, breast cancer cell lysate-pulsed DC (as APC stimulators) and autologous T cells (as responders) will be initiated in year 2 of the funded project]. Lastly, and most important to the studies proposed in Technical Objective 2, TNF-α-treated DC were also found to be potently chemoattracted by the chemokines MCP-1 (Figure 10) and lymphotactin (Figure 11) when evaluated in standard microchemotaxis assays. (We also plan to evaluate additional recombinant chemokines, namely RANTES, MIP-1α, and MIP-1β, for effects on DC in year 2 of our funded project). **Thus, we have been successful in optimizing the generation of human peripheral blood-derived DC with enhanced functional properties as originally proposed in Technical Objective 1 of our funded grant application. Moreover, we have shown that autologous DC can be successfully derived from advanced breast cancer patients, which, upon pulsing, can stimulate potent tetanus toxoid- and KLH-specific proliferative responses by purified T**

cells obtained from these same patients. This latter finding considerably lessons the potential concern of a compromised immune system in advanced breast cancer patients (as a result of multiple chemotherapy/radiation therapy regimens that are also immunosuppressive) hampering attempts to clinically develop and utilize dendritic cell-based vaccines in breast tumor immunization approaches in vivo.

(b) Technical Objective 2: As stated in the Introduction, the primary goal of the funded application is to enhance the capacity of breast tumor-pulsed DC to trigger antitumor immune responses by including chemokine gene-modified fibroblasts to potently and selectively recruit specific immune cell subsets. The research directed in year one under Technical Objective 2 emphasized the construction of expression vectors containing the cDNAs encoding for five (5) distinct chemokines, transfection/transduction of fibroblasts, and analysis of chemokine production by molecular, ELISA, and/or functional assays. **Our efforts have been highly successful.** Our initial attempts to transfect fibroblasts with chemokine gene-containing expression plasmids were not fruitful, since transfection efficiencies and levels of chemokine production by the transfected fibroblasts were both low. In order to overcome these deficiencies, we thus modified our original proposed strategy and focused our efforts on constructing high-efficiency retroviral vectors. We have now successfully introduced the cDNAs encoding for lymphotactin (Ltn), RANTES, as well as macrophage inflammatory protein-1 beta (MIP-1β) into the MFG-based retroviral vector backbone (Figure 12). We are also currently completing the vector construction to express the monocyte chemotactic protein-1 (MCP-1) and MIP-1α; given our success with the three other aforementioned chemokines we do not foresee any difficulties in doing so. Chemokine cDNAs were amplified by PCR and were introduced into the MFG-based retroviral vector backbone with the start codon of the cDNA inserted precisely at the start codon of the deleted env gene (at the NcoI site). The cloned chemokine cDNAs were then transcribed from the promoter/enhancer sequences in the retroviral long terminal repeat (LTR) sequence. Expression plasmids containing the MFG/chemokine cDNA constructs were transferred into both the amphotropic ψNX-A and ecotropic ψNX-E high-efficiency, transient packaging cell lines (obtained from Dr. G. Nolan, Stanford University). High-titer retroviral supernatant from these producer cells was used to infect murine NIH-3T3 cells and autologous human fibroblasts. Genomic DNA of these transduced target cells was analyzed by PCR with two primers, one of which is located in the MFG 3'LTR and the second in the chemokine cDNA. The PCR results showed the integration of the MFG retroviral vectors with either Ltn, RANTES, or MIP-1β cDNA sequences (Figure 13). The transgene expression of the chemokines in NIH-3T3 cells and autologous fibroblasts was examined by RT-PCR; RT-PCR fragments were strongly detected in the MFG/chemokine cDNA transduced cells compared to a complete lack of RT-PCR products in both control unmodified, parental cells and MFG backbone alone transduced cells (not shown).

We then evaluated the level of production of chemokines in the culture supernatants of plated transduced fibroblasts by either ELISA or functional microchemotaxis assays (Figures 14 and 15). Significant levels of RANTES and MIP-β chemokine were produced from transduced breast cancer patient's fibroblasts (Figure 14) and 3T3 cells (Figure 15, lower panel). Since an ELISA was not available for Ltn, we performed microchemotaxis assays with supernatant harvested from transduced 3T3 cells and found selective chemoattraction of $CD4^+$ and to a greater extent $CD8^+$ T cells purified from the peripheral blood of an advanced breast cancer patient (Figure 15, upper panel). **Collectively, these results demonstrated the successful expression of three distinct chemokine cDNAs constructed into the MFG-based retroviral vector. Moreover, transduced fibroblasts have produced significant levels of biologically active chemokines from the introduced transgene(s).** Thus, these chemokine-gene modified fibroblasts will now allow us to conduct our next proposed series of studies to combine them with tumor lysate-pulsed dendritic cells in an attempt to detect, attract, and augment specific, antigen-reactive T cells in vitro from breast cancer patients (Technical Objective 3).

Conclusion/Significance

The significance of our research lies in the potential to develop a new, innovative molecular vaccine strategy for eventual use in breast cancer patients that employs chemokine gene-modified fibroblasts combined with tumor-pulsed dendritic cells to both recruit/concentrate relevant immune populations at the vaccination site (by secreted chemokines) as well as to activate the recruited T cells by potent presentation of tumor-associated antigens (by dendritic cells). This strategy may prove to be a highly effective means of both detecting and augmenting the immune response to poorly-immunogenic breasttumors that ultimately leads to tumor eradication.

Plans

The upcoming year will be the second year of the four-year support for this research project. **No significant changes are anticipated with respect to the experimental design and methods of the Technical Objectives.** We plan to complete the construction of the chemokine-gene containing retroviral vectors and optimize the conditions for transduction of fibroblasts from breast cancer patients to achieve the highest level of production of the relevant chemokine(s). We will also optimize the pulsing step of breast tumor lysates onto "cytokine-driven" autologous DC to generate the most potent elicitation of specific T cell reactivity in vitro. We will then initiate the combination approach of chemokine gene-modified fibroblasts and tumor-pulsed DC in vitro and begin to dissect the underlying mechanisms of antitumor T cell reactivities and chemoattraction observed. We also hope to increase our knowledge of the mechanisms of T cell activation, recognition, and destruction of poorly-immunogenic tumors. Finally, we believe our experimental data will be sufficiently relevant to warrant the design and execution of a spin-off phase I clinical trial in patients with advanced breast cancer.

References

1. Rosenberg SA: Principles and applications of biologic therapy. In: Cancer: Principles and Practice of Oncology. DeVita Jr VT, Hellman S, Rosenberg SA (eds.), JB Lippincott Co., Philadelphia, p. 293, 1993.
2. Rosenberg SA: Cell transfer therapy: Clinical applications. In: Biologic Therapy of Cancer. DeVita Jr VT, Hellman S, Rosenberg SA (eds.), JB Lippincott Co., Philadelphia, p. 487, 1994.
3. Mulé JJ, Shu S, Schwarz SL, Rosenberg SA: Adoptive immunotherapy of established pulmonary metastases with LAK cells and recombinant interleukin-2. Science 225: 1587, 1984.
4. Barth, Jr RJ, Bock SN, Mulé JJ, Rosenberg SA: Unique murine tumor associated antigens identified by tumor infiltrating lymphoctyes. J. Immunol. 144: 1531, 1990.
5. Schwartzentruber DJ, Solomon D, Rosenberg SA, Topalian SL: Characterization of lymphoctyes infiltrating human breast cancer: specific immune reactivity detected by measuring cytokine secretion. J. Immunother. 12: 1 - 12, 1992.
6. Jerome KR, Domenech N, Finn OJ: Tumor-specific cytotoxic T cell clones from patients with breast and pancreatic adenocarcinoma recognize EBV-immortalized B cells transfected with polymorphic epithelial mucin complementary DNA. J. Immunol. 151: 1654-1662, 1993.
7. Magarian-Blander J, Domenech N, Finn OJ: Specific and effective T-cell recognition of cells transfected with a truncated human mucin cDNA. Ann. N.Y. Acad. Sci. 690: 231-43, 1993.
8. Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, Eberlein TJ: Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc. Natl. Acad. Sci. USA 92: 432-436, 1995.
9. Sondak VK, Wagner PD, Shu S, Chang AE: Suppressive effect of visceral tumor on the generation of antitumor T cells for adoptive immunotherapy. Arch. Surg. 126: 442, 1991.
10. Ochoa AC, Longo DL: Alteration of signal transduction in T cells from cancer patients. In: Important Advances in Oncology 1995. DeVita Jr VT, Hellman S, Rosenberg SA (eds.), JB Lippincott Co., Philadelphia, p. 43, 1995.

11. Salvadori S, Gansbacher B, Pizzimenti AM, Zier KS: Abnormal signal transduction by T cells of mice with parental tumors is not seen in mice bearing IL-2 secreting tumors. J. Immunol. 153: 5176-5182, 1994.
12. Coulie PG, Somville M, Lehmann F, Hainaut P, Brasseur F, Devos A, Boon T: Precursor frequency analysis of human cytolytic T Lymphoctyes directed against autologous melanoma cells. Int. J. Cancer 50: 289, 1992.
13. Restifo NP, Esquivel F, Dawakami Y, Yewdell JW, Mulé JJ, Rosenberg SA, Bennink JR: Identification of human cancers deficient in antigen processing. J. Exp. Med. 177: 265-272, 1993.
14. Hwu P, Rosenberg SA: Gene therapy using lymphocyte modification. In: Biologic Therapy of Cancer. DeVita Jr VT, Hellman S, Rosenberg SA (eds.), JB Lippincott Co., Philadelphia, p. 727, 1994.
15. Hwu P, Shafer GE, Treisman J, Schindler DG, Gross G, Cowherd R, Rosenberg SA, Eshhar Z: Lysis of ovarian cancer cells by human lymphoctyes redirected with a chimeric gene composed of an antibody varable region and the Fc receptor gamma chain. J. Exp. Med. 178: 361, 1993.
16. Houghton AN: Cancer antigens: Immune recognition of self and altered self. J. Exp. Med. 180: 1, 1994.
17. Tepper RI, Mulé JJ: Experimental and clinical studies of cytokine gene-modified tumor cells. Human Gene Ther. 5: 153, 1994.
18. Kerr WG, Mulé JJ: Gene therapy: Current status and future prospects. J. Leuko. Biol. 56: 210, 1994.
19. Shiloni E, Karp SE, Custer MC, Shilyansky J, Restifo NP, Rosenberg SA, Mulé JJ: Retroviral transduction of interferon-gamma cDNA into a nonimmunogenic murine fibrosarcoma: Generation of T cells in draining lymph nodes capable of treating established parental metastatic tumor. Cancer Immunol. Immunother. 34: 386, 1993.
20. Romani N, Kampgen E. Koch F, Heufler C, Schuler G: Dendritic cell production of cytokines and responses to cytokines. Int. Rev. Immunol. 6:151, 1990.
21. Katz SI, Tamaki K, Sachs DH: Epidermal Langerhans cells are derived from cells originating in the bone marrow. Nature 282: 324, 1979.
22. Steinman RM, Gutchinor B, Witmer MD, Nussenzweig MC: Dendritic cells are principal stimulators of the primary mixed leukocyte reaction in mice. J. Exp. Med. 157: 613-627, 1983.
23. Stingl G, Bergstresser PR: Dendritic cells: a major story unfolds. Immunol. Today 16: 330-333, 1995.
24. Croft M, Bradley LM, Swain SL: Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. J. Immunol. 152: 2675, 1994.
25. Crowley M, Inaba K, Steinman RM: Dendritic cells are the principal cells in mouse spleen bearing immunogenic fragments of foreign proteins. J. Exp. Med. 172: 383,1990.
26. Girolomoni G, Simon JC, Bergstresser PR, Cruz, Jr. PD: Freshly isolated spleen dendritic cells and epidermal Langerhans cells undergo similar phenotypic and functional changes during short-term culture. J. Immunol. 145: 2820, 1990.
27. Hauser C, Katz SI: Activation and expansion of hapten-and protein-specific T helper cells from nonsensitized mice. Proc. Natl. Acad. Sci. USA 85: 5625, 1988.
28. Mehta-Damani A, Markowicz S, Engleman EG: Generation of antigen-specific $CD8^+$ CTL from naive precursors. J. Immunol. 153: 996, 1994.
29. Grabbe S, Bruvers S, Gallo RL, Knisely TL, Nazareno R: Tumor antigen presentation by murine epidermal cells. J. Immunol. 146: 3656, 1991.
30. Grabbe S, Beissert S, Schaw T, Granstein RD: Dendritic cells as initiators of tumor immune responses: a possible strategy for tumor immunotherapy. Immunol. Today 16: 117-121, 1995.
31. Flamand V, Sornasse T, Thielemans K, Demanet C, Bakkus M, Bazin H, Thielemans F, Leo O, Urbain J, Moser M: Murine dendritic cells pulsed *in vitro* with tumor antigen induce tumor resistance *in vivo*. Eur. J. Immunol. 24: 605-610, 1994.
32. Grabbe S, Bruvers S, Gallo RL, Knisely TL, Nazareno R, Granstein RD: Tumor antigen presentation by murine epidermal cells. J. Immunol. 146: 3656-3661, 1991.

33. Greenberg PD, Kern DE, Cheever MA: Therapy of disseminated murine leukemia with cyclophosphamide and immune Lyt-1^+ 2^- T cells. Tumor eradication does not require participation of cytotoxic T cells. J. Exp. Med. 161: 1122, 1985.
34. Kahn M, Sugawara H, McGowan P, Okuno K, Nagoya S, Hellstrom KE, Hellstrom I and Greenberg P: $CD4^+$ T cell clones specific for the human p97 melanoma-associated antigen can eradicate pulmonary metastases from a murine tumor expressing the p97 antigen. J. Immunol. 146: 3235-3241, 1991.
35. Fujiwara H, Fukuzawa M, Yoshioka T, Nakajima H, Hamaoka T: The role of tumor-specific Lyt-1^+ 2^- T cells in eradicating tumor cells in vivo. Lyt-1^+ 2^- T cells do not necessarily require recruitment of host's cytotoxic T cell precursors for implementation of in vivo immunity. J. Immunol. 133: 1671, 1984.
36. Topalian SL, Rivoltini L, Mancini M, Ng J, Hartzman RJ, Rosenberg SA: Melanoma-specific $CD4^+$ T lymphocytes recognize human melanoma antigens processed and presented by Epstein-Barr virus-transformed B cells. Int. J. Cancer 58: 69-79, 1994.
37. Topalian SL, Rivoltini L, Mancini M, Markus NR, Robbins PF, Kawakami Y, Rosenberg SA: Human $CD4^+$ T cells specifically recognize a shared melanoma-associated antigen encoded by the tyrosinase gene. Proc. Natl. Acad. Sci. USA 91: 9461-9465, 1994.
38. Matis LA, Ruscetti SK, Longo DL, Jacobson S, Brown EJ, Zinn S, Kruisbeek AM: Distinct proliferative T cell clonotypes are generated in response to a murine retrovirus-induced syngeneic T cell leukemia: viral gp70 antigen-specific $MT4^+$ clones and Lyt-2^+ cytolytic clones which recognize a tumor-specific cell surface antigen. J. Immunol. 135: 703-713, 1985.
39. Cohen PJ, Cohen PA, Rosenberg SA, Katz SI, Mulé JJ: Murine epidermal Langerhans cells and splenic dendritic cells present tumor-associated antigens to primed T cells. Eur. J. Immunol. 24: 315-319, 1994.
40. Cohen PA, Cohen PJ, Rosenberg SA, Mulé JJ: $CD4^+$ T-cells from mice immunized to syngeneic sarcomas recognize distinct, non-shared tumor antigens. Cancer Research 54: 1055-1048, 1994.
41. Cohen PA, Kim H, Fowler DH, Gress RE, Jakobsen MK, Alexander RB, Mulé JJ, Carter C, Rosenberg SA: Use of Interleukin-7, Interleukin-2, and Interferon-γ to propagate $CD4^+$ T cells in culture with maintained antigen specificity. J. Immunother. 14: 242-252, 1993.
42. Sallusto F, Lanzavecchia A: Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus Interleukin 4 and downregulated by tumor necrosis Factor α. J. Exp. Med. 179: 1109- 1118.
43. Romani N, Gruner S, Brang D, Kämpgen E, Lenz A, Trockenbacker B, Konwalinka G, Fritsch PO, Steinman RM, Schuler G: Proliferating dendritic cell progenitors in human blood. J. Exp. Med. 180: 83-93, 1994.
44. Lu L, Woo J, Rao AS, Li Y, Watkins SC, Qian S, Starzl TI, Demetris AJ, Thomson AW: Propagation of dendritic cell progenitors from normal mouse liver using granulocyte/macrophage colony-stimulating factor and their maturational development in the presence of type-1 collagen. J. Exp. Med. 179: 1823-1834, 1994.
45. Kämpgen E, Koch F, Heufler C, Eggert A, Gill LL, Gillis S, Dower SK, Romani N, Schuler G: Understanding the dendritic cell lineage through a study of cytokine receptors. J. Exp. Med. 179: 1767-1776, 1994.
46. Grabbe S, Bruvers S, Lindgren AM, Hosoi J, Tan KC, Granstein RD: Tumor antigen presentation by epidermal antigen-presenting cells in the mouse: modulation by granulocyte-macrophage colony-stimulating factor, tumor necrosis factor α, and ultraviolet radiation. J. Leukocyte Biol. 52: 209-217, 1992.
47. Porgador A, Gilboa E: Bone marrow-generated dendritic cells pulsed with a class I-restricted peptide are potent inducers of cytotoxic T lymphocytes. J. Exp. Med. 182: 255-260, 1995.
48. Bernhard H, Disis ML, Heimfeld S, Hand S, Gralow JR, Cheever MA: Generation of immunostimulatory dendritic cells from human CD34+ hematopoietic progenitor cells of the bone marrow and peripheral blood. Cancer Research 55: 1099-1104, 1995.

49. Barth, Jr. RJ, Mulé JJ: Cytokine gene transfer into tumor cells: animal models. In: Gene Transfer: Scientific Advances and Clinical Developments. MK Brenner and RC Moen (eds). Marcel Dekker, New York 1995. (in press).
50. Schall TJ: The Chemokines. In: The Cytokine Handbook. A.W. Thomson (ed.). Academic Press, New York, pp. 419-460, 1994.
51. Schall TJ, Bacon K, Toy KJ, Goeddel DV: Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. Nature 347: 669, 1990.
52. Schall TJ, Bacon K, Camp RDR, Kaspari JW, Goeddel DV: Human macrophage inflammatory protein α (MIP-1α) and MIP-1β chemokines attract distinct populations of lymphocytes. J. Exp. Med. 177: 1821, 1993.
53. Carr MW, Roth SJ, Luther E, Rose SS, Springer TA: Monocyte chemoattractant protein-1 is a major T lymphocyte chemoattractant. Proc. Natl. Acad. Sci. USA 91:3652-3656, 1994.
54. Turner L, Ward SG, Westwick J: RANTES-Activated human T lymphocytes: A role for phosphoinositide 3-kinase. J. Immunol. 155: 2437-2444, 1995.
55. Bacon KB, Premack BP, Gardener P, Schall TJ: Activation of dual T cell signaling pathways by the chemokine RANTES. Science 1995. (in press).
56. Taub DD, Conlon K, Lloyd AR, Oppenheim JJ, Kelvin DJ: Selective chomo-attraction of CD4 and CD8 subsets of human T cells by macrophage inflammatory protein-1α and 1β. Science 60: 355-358, 1993.
57. Kelner GS, Kennedy J, Bacon KB, Kleyensteuber S, Largaespada DA, Jenkins NA, Copeland NG, Bazan JF, Moore KW, Schall TJ, Zlotnik A: Lymphotactin: a cytokine that represents a new class of chemokine. Science 266: 1395, 1994.
58. Kennedy J, Kelner GS, Kleyensteuber S, Schall, TJ, Weiss MC, Yssel H, Schneider PV, Cocks BG, Bacon K, Zlotnick A: Molecular cloning and functional characterization of human lymphotactin. J. Immunol. 155: 203-209, 1995.
59. Mulé JJ, Custer M, Averbook B, Yang JC, Weber JS, Goeddel DV, Rosenberg SA, Schall TJ: RANTES secretion by gene modified tumor cells results in loss of tumorigenicity *in vivo*: role of immune cell subpopulations. (submitted, 1995)
60. Laning J, Kawasaki H, Tanaka E, Luo Y, Dorf ME: Inhibition of *in vivo* tumor growth by the β chemokine, TCA3. J. Immunol. 153: 4625-4635, 1994.
61. Sornasse T, Flamand V, DeBecker G, Bazin H, Thielemans F, Thielemans K, Urbain J, Leo O, Moser M: Antigen-pulsed dendritic cells can efficiently induce antibody response in vivo. J. Exp. Med. 175: 15, 1992.
62. Freudenthal PS, Steinman RM: The distinct surface of human blood dendritic cells, as observed after an improved isolation method. Proc. Natl. Acad. Sci. USA 87: 7698-7702, 1990.
63. O'Doherty V, Peng M, Gezelter S, Swiggard WJ, Betjes M, Bhardwaj N, Steinman RM: Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. Immunology 82: 487, 1994.

Bibliography - Meeting Abstract

Chen, BG, Smith, JD, Geiger, JD, and Mulé, JJ: TNFα expands human dendritic cells cultured in serum-free medium and enhances soluble antigen presentation to CD4+ T cells. J. Allergy Clin. Immunol. 99: S450 (1827), 1997 (AAI 1997 meeting abstract).

APPENDICES: DATA FIGURES AND TABLES

Figure 1

Responsiveness of primary CD4 T cells from EE breast cancer patient to different antigen pulsed DC

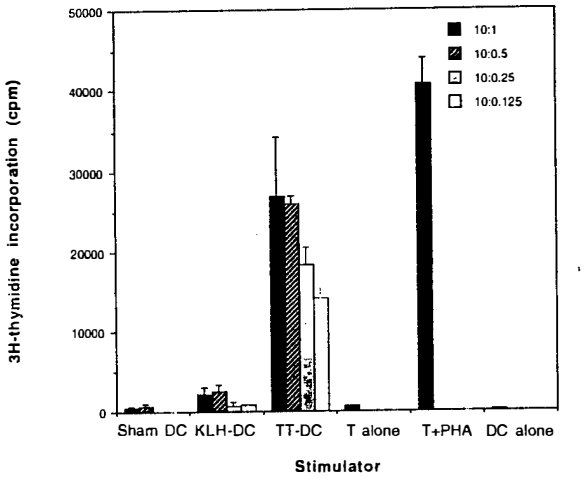
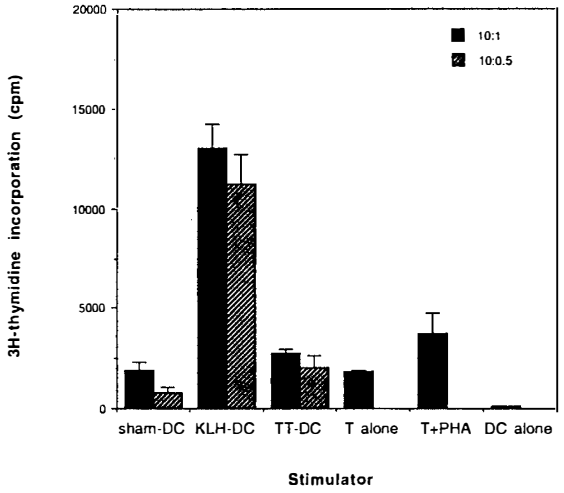


Figure 2

Responsiveness of third KLH-DC stimulated CD4 T cells from EE breast cancer patient to different antigen pulsed DC



APPENDICES: DATA FIGURES AND TABLES

Figure 3

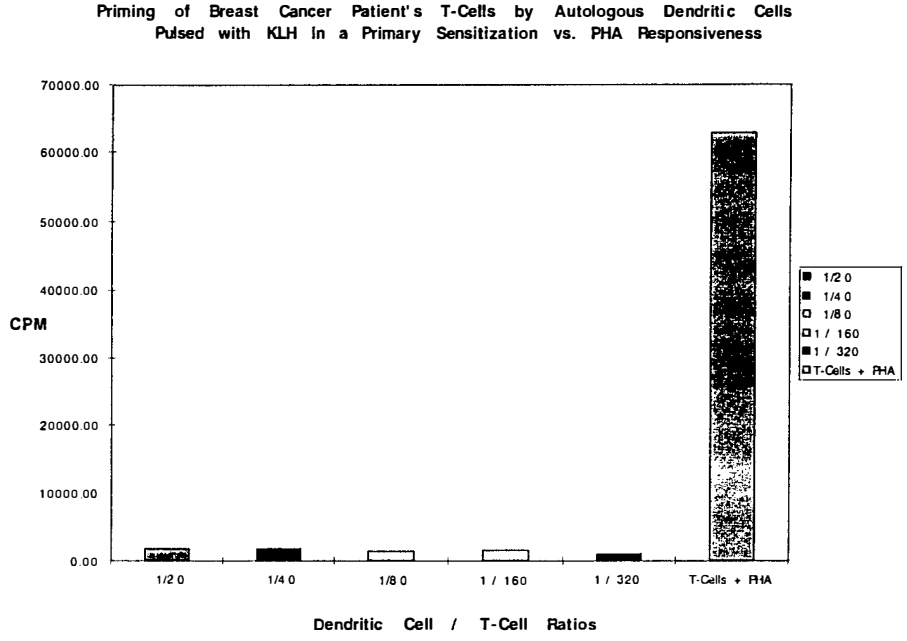
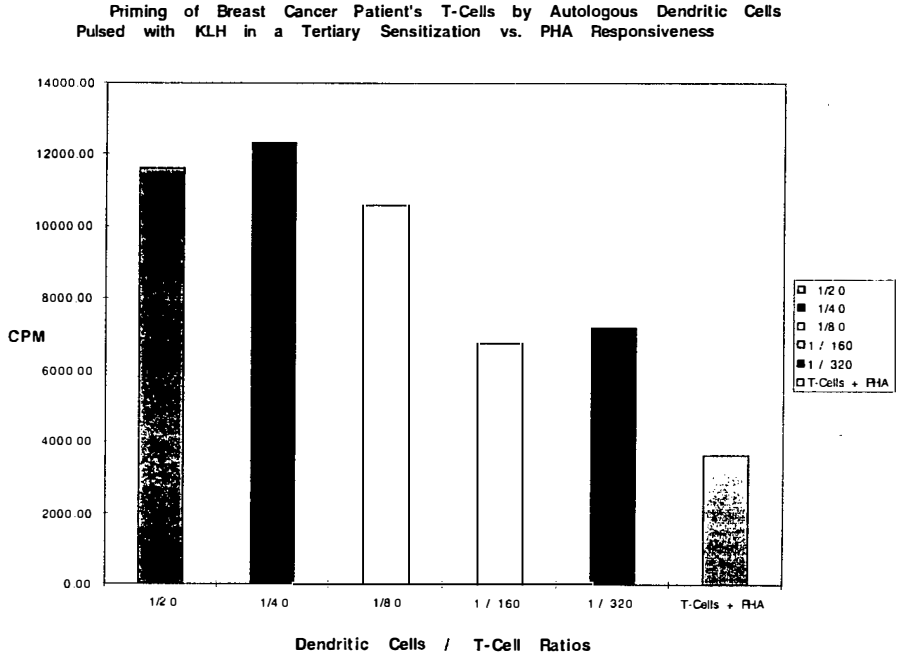


Figure 4



APPENDICES: DATA FIGURES AND TABLES

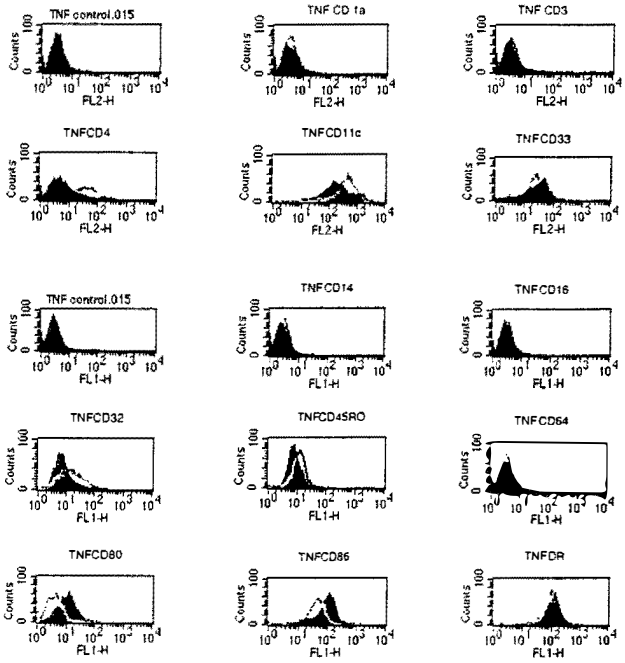
Table1. Yield of dendritic cells cultured with or without TNFα

Expt.	(-) TNFα (X 10^5)*	(+) TNFα (X 10^5)*
1	1.0	2.55
2	1.9	2.85
3	1.15	2.5
4	1.3	3.5
5	1.5	2.8
6	1.1	2.5
7	1.3	2.7
X ± SD	1.32 ± 0.3	2.77 ± 0.35

* represents the number of day 14 harvested dendritic cells per 5 X 10^6 PBMC.

Phenotypic changes of DCs cultured for 7 days with TNFα compared to non-TNFα DCs.

Figure 5



APPENDICES: DATA FIGURES AND TABLES

Figure 6

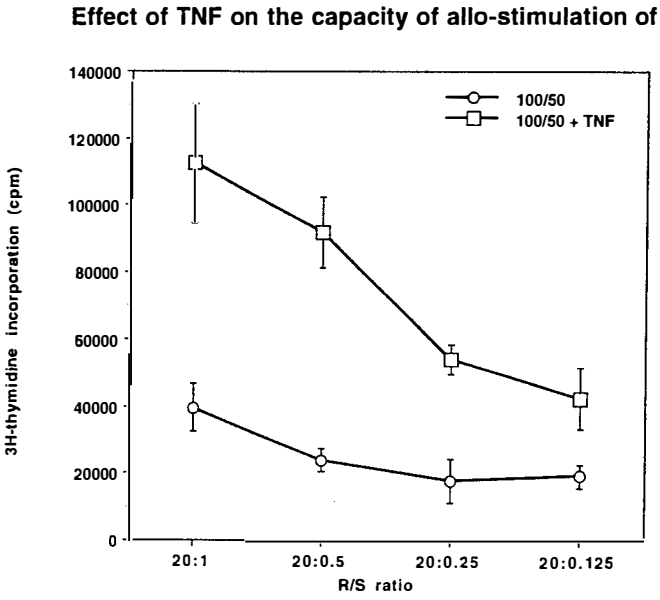
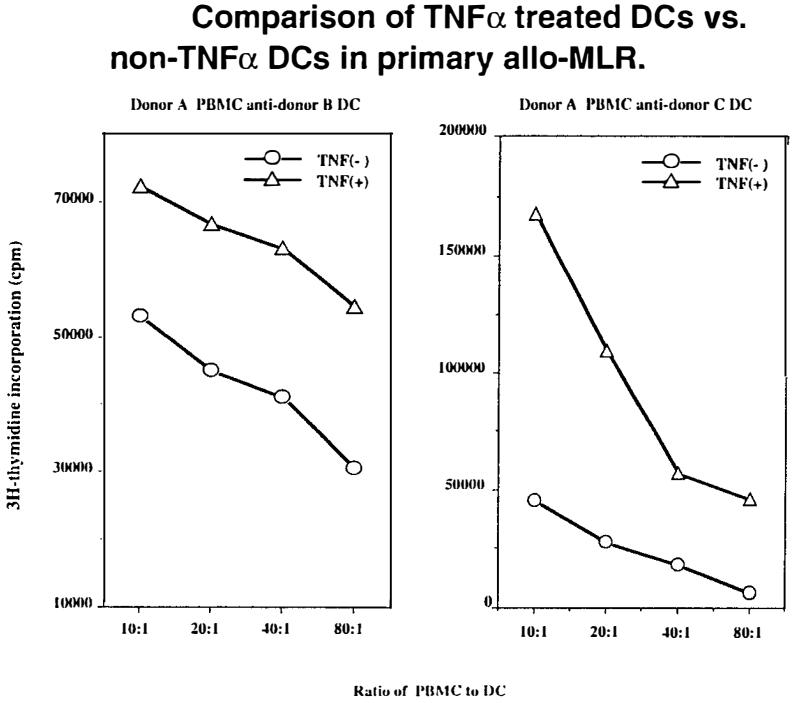


Figure 7



APPENDICES: DATA FIGURES AND TABLES

Figure 8

Comparison of TNFα cultured DCs vs. non-TNFα DCs for presentation of soluble antigen: antigen titration.

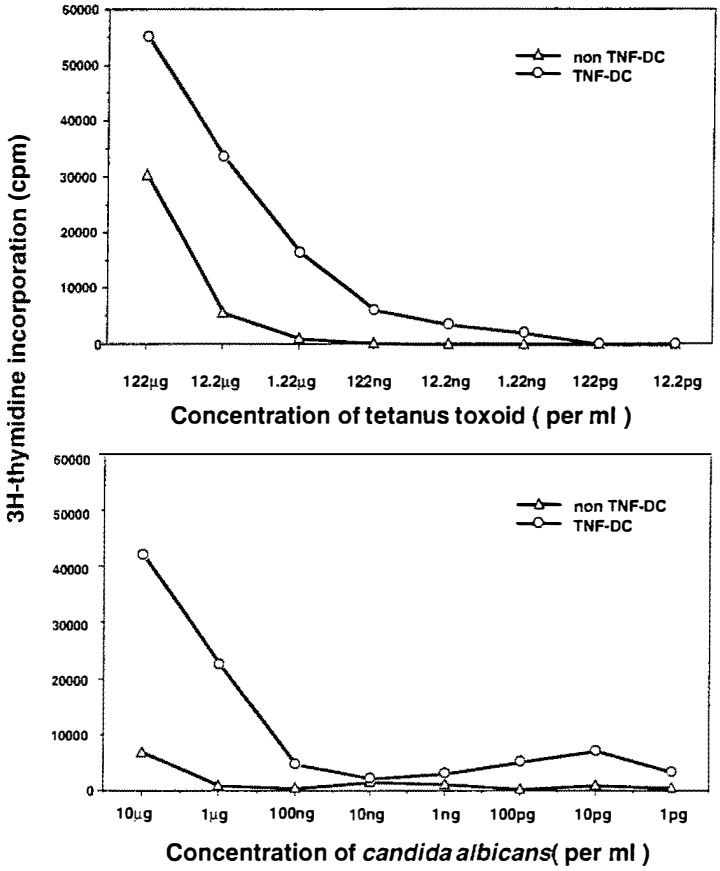
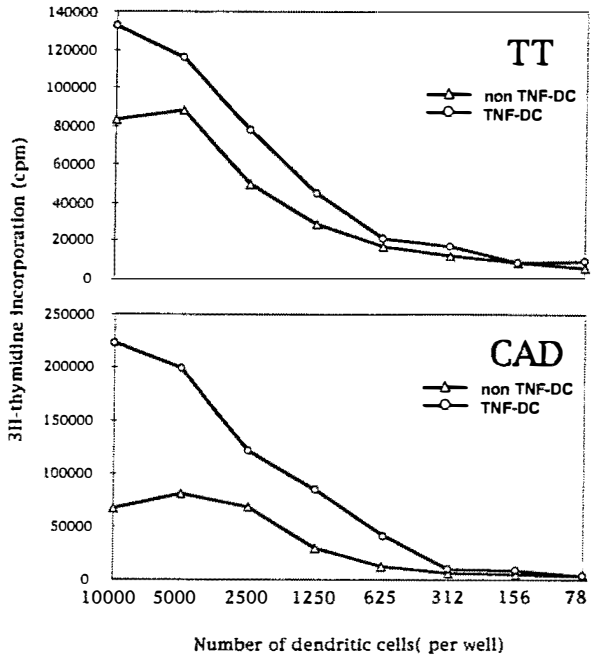


Figure 9

Comparison of TNFα cultured DCs vs. non-TNFα DCs for presentation of soluble antigen: DC titration.



APPENDICES: DATA FIGURES AND TABLES

Figure 10

The Chemotactic response of TNF treated DC

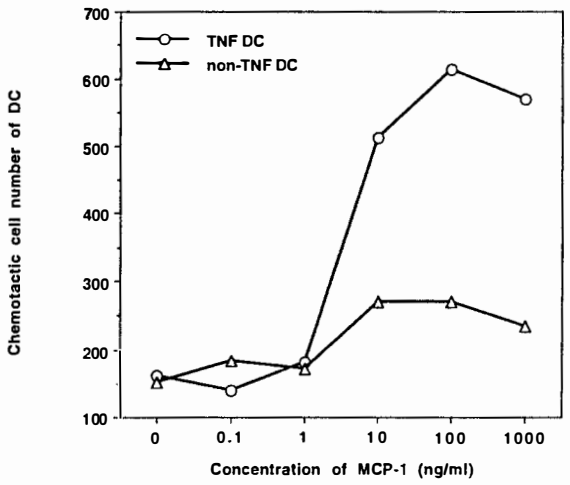


Figure 11

The Chemotactic response of TNF treated DC

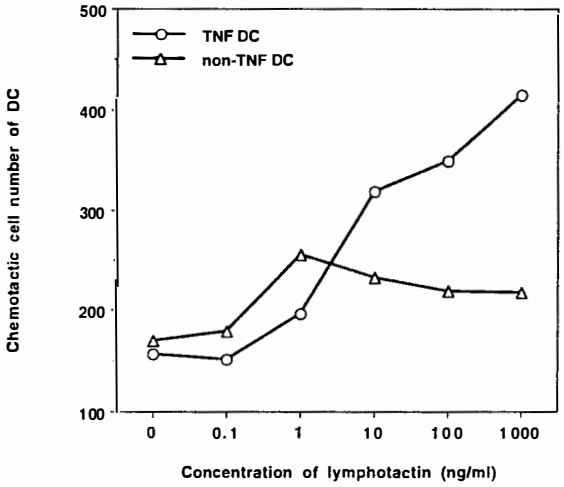
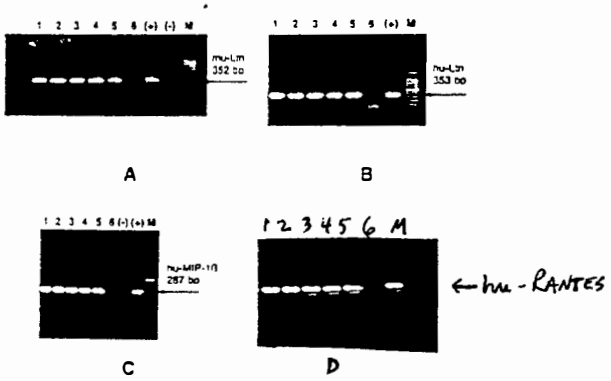


Figure 12

Detection of chemokine cDNA in MFG-based retroviral vector determined by PCR.



A) Plasmid MFG/mo-lymphotactin. lane 1-5: 5 clones with MFG/mo-lymphotactin. plasmid, lane 6: MFG-based retroviral backbone, M: ϕx174DNA-HaeIII.
B) Plasmid MFG/hu-lymphotactin. lane 1-5: 5 clones with MFG/hu-lymphotactin, lane 6: MFG-based retroviral backbone, M: 100 bp DNA ladder.

C) Plasmid MFG/hu-MIP-1β, lane 1-5: 5 clones with MFG/hu-MIP-1β, lane 6: MFG-based retroviral backbone, M: 100bp DNA ladder.

D) Plasmid MFG/hu-Rantes. lane 1-5: 5 clones with MFG/hu-MIP-1β, lane 6: MFG-based retroviral backbone. M: 100bp DNA ladder.

(+) positive control using the constructed pBS/chemokine plasmid as templates,
(-) negative control without templates

APPENDICES: DATA FIGURES AND TABLES

Figure 13

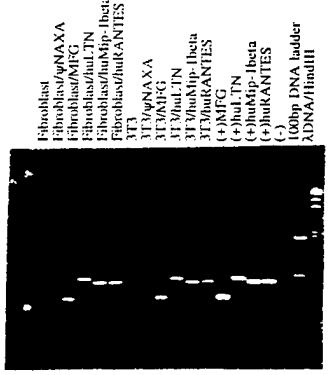


Figure 14

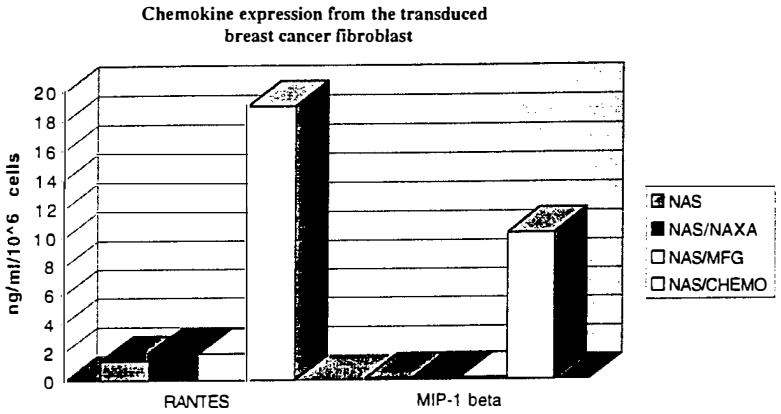
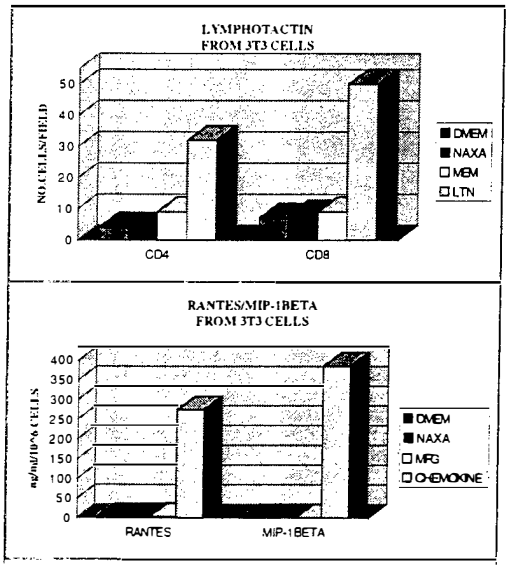


Figure 15



PII Redacted

Principal Investigator/Program Director *(Last, first, middle)*: Mule', James, J.

PERSONNEL REPORT

All Personnel for the Current Budget Period

Name	Degree(s)	[redacted]	Role on Project *(e. g. PI, Res. Assoc.)*	[redacted]	Annual % Effort
James J. Mulé	Ph.D.		P.I.		10%
Alfred E. Chang	M.D.		Co-Invest.		5%
Vernon K. Sondak	M.D.		Co-Invest.		5%
Stephen P. Ethier	Ph.D.		Co-Invest.		5%
Yijun Shi	M.Sci.		Res. Assoc. II		75%